

Experimental SARS-CoV-2 Infection of Bank Voles

Lorenz Ulrich,¹ Anna Michelitsch,¹ Nico Halwe,¹ Kerstin Wernike, Donata Hoffmann, Martin Beer

After experimental inoculation, severe acute respiratory syndrome coronavirus 2 infection was confirmed in bank voles by seroconversion within 8 days and detection of viral RNA in nasal tissue for up to 21 days. However, transmission to contact animals was not detected. Thus, bank voles are unlikely to establish effective transmission cycles in nature.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) led to a global pandemic in the human population within months after its first reporting (1). Potential wildlife reservoirs of SARS-CoV-2 remain unknown; susceptibility of various animal species has been described (2,3). Among rodent species, the Syrian hamster (*Mesocricetus auratus*) (4) and the North American deer mouse (*Peromyscus maniculatus*) (A. Fagre et al., unpub. data, <https://doi.org/10.1101/2020.08.07.241810>; B.D. Griffin et al., unpub. data, <https://doi.org/10.1101/2020.07.25.221291>), both *Cricetidae* species, have proved to be highly susceptible. These rodents transmit SARS-CoV-2 to co-housed contact animals and therefore are likely to develop effective infection chains, which could result in independent SARS-CoV-2 transmission cycles in nature and sequential reintroduction to the human population (4; B.D. Griffin et al., unpub. data, <https://doi.org/10.1101/2020.07.25.221291>). In Europe, bank voles (*Myodes glareolus*) are a widespread *Cricetidae* species (5). We aimed to characterize SARS-CoV-2 infection in bank voles and their ability to maintain sustainable infection chains.

We intranasally inoculated 9 bank voles with SARS-CoV-2 strain Muc-IMB-1 and, 24 hours later, co-housed 1 contact animal with each of 3 groups of 3 inoculated animals (donor-recipient ratio [d:r] 3:1). We took swab samples regularly from all animals (Appendix, <https://wwwnc.cdc.gov/EID/article/27/4/20-4945-App1.pdf>); we euthanized 1 or

2 animals at predefined times (Appendix). One bank vole did not survive initial anesthesia for inoculation.

Neither inoculated nor contact animals showed clinical signs during the study. We detected seroconversion for all directly inoculated animals euthanized 8, 12, and 21 days postinfection (dpi), whereas the animals euthanized 4 dpi and the contact animals were all clearly seronegative for SARS-CoV-2 antibodies in an already validated indirect multispecies ELISA based on the receptor-binding domain (6).

All directly inoculated bank voles tested positive for SARS-CoV-2 by quantitative reverse transcription PCR (qRT-PCR) by oral and rhinarium swab specimens at 2 dpi. At 4 dpi, 5 of these 8 animals were positive by oral swab specimen; 2 were also positive by rhinarium swab specimen. On both sampling days, rectal swab specimens of 2 animals tested positive for SARS-CoV-2 by qRT-PCR. Groupwise collected fecal samples also tested positive by qRT-PCR at 2 and 4 dpi. All swabs collected 8, 12, and 16 dpi from directly inoculated animals and every swab from the co-housed contact animals tested negative by qRT-PCR (Table; Figure).

Two animals were euthanized at 4 dpi; nasal conchae, trachea, lung, and olfactory bulb samples tested positive for SARS-CoV-2 RNA by qRT-PCR (quantification cycle [C_q] 25.45–37.15). One animal showed viral genome in cerebrum and cerebellum samples, whereas the spleen sample from the other animal was positive for the viral genome. At 8 dpi another 2 animals were euthanized; both exhibited viral RNA only within the nasal conchae. The animal euthanized at 12 dpi was negative in all collected tissue samples. Nasal conchae of 3 inoculated animals euthanized at 21 dpi tested positive by qRT-PCR (C_q values 34.78, 34.97, 36.25), whereas all 3 contact animals euthanized at the same time tested negative in the nasal conchae.

Reisolation of viable virus from tissue materials in cell culture (Vero E6) was successful for 1 nasal conchae sample taken at 4 dpi. However, isolation

Author affiliation: Friedrich-Loeffler-Institut, Greifswald–Insel Riems, Germany

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¹These authors contributed equally to this article.

Table. Quantitative reverse transcription PCR results of swab sampling for all inoculated and contact bank voles in experimental study of SARS-CoV-2 transmission*

Box	Status	Swab	-1 dpi	2 dpi	4 dpi	8 dpi	12 dpi	16 dpi
Box 1	Inoculated	Oral	Neg	32.45	Neg	Neg	Neg	Neg
		Nasal	Neg	32.29	Neg	Neg	Neg	Neg
		Rectal	Neg	Neg	Neg	Neg	Neg	Neg
	Inoculated	Oral	Neg	NA	NA	NA	NA	NA
		Nasal	Neg	NA	NA	NA	NA	NA
		Rectal	Neg	NA	NA	NA	NA	NA
	Inoculated	Oral	Neg	32.09	28.16	Neg	Neg	Neg
		Nasal	Neg	31.72	34.03	Neg	Neg	Neg
		Rectal	Neg	36.54	36.39	Neg	Neg	Neg
	Contact	Oral	Neg	Neg	Neg	Neg	Neg	Neg
		Nasal	Neg	Neg	Neg	Neg	Neg	Neg
		Rectal	Neg	Neg	Neg	Neg	Neg	Neg
	Collected feces		Neg	36.58	37.66	Neg	Neg	Neg
Box 2	Inoculated	Oral	Neg	29.40	32.41	NA	NA	NA
		Nasal	Neg	32.68	34.72	NA	NA	NA
		Rectal	Neg	Neg	Neg	NA	NA	NA
	Inoculated	Oral	Neg	30.46	32.54	Neg	NA	NA
		Nasal	Neg	32.30	Neg	Neg	NA	NA
		Rectal	Neg	36.67	Neg	Neg	NA	NA
	Inoculated	Oral	Neg	32.72	37.07	Neg	Neg	Neg
		Nasal	Neg	34.74	Neg	Neg	Neg	Neg
		Rectal	Neg	Neg	Neg	Neg	Neg	Neg
	Contact	Oral	Neg	Neg	Neg	Neg	Neg	Neg
		Nasal	Neg	Neg	Neg	Neg	Neg	Neg
		Rectal	Neg	Neg	Neg	Neg	Neg	Neg
	Collected feces		Neg	36.06	36.65	Neg	Neg	Neg
Box 3	Inoculated	Oral	Neg	30.98	Neg	Neg	NA	NA
		Nasal	Neg	31.63	Neg	Neg	NA	NA
		Rectal	Neg	Neg	Neg	Neg	NA	NA
	Inoculated	Oral	Neg	30.66	34.32	NA	NA	NA
		Nasal	Neg	34.52	Neg	NA	NA	NA
		Rectal	Neg	Neg	34.89	NA	NA	NA
	Inoculated	Oral	Neg	32.64	Neg	Neg	Neg	NA
		Nasal	Neg	35.46	Neg	Neg	Neg	NA
		Rectal	Neg	Neg	Neg	Neg	Neg	NA
	Contact	Oral	Neg	Neg	Neg	Neg	Neg	Neg
		Nasal	Neg	Neg	Neg	Neg	Neg	Neg
		Rectal	Neg	Neg	Neg	Neg	Neg	Neg
	Collected feces		Neg	36.62	37.02	Neg	Neg	Neg

*Positive results are given as quantification cycle values. dpi, days postinoculation; NA, not applicable; Neg, negative; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

from samples with Cq >28 failed, in line with findings of other groups (3,7).

Overall, bank voles proved to be susceptible to infection with SARS-CoV-2 but did not transmit the virus to co-housed direct contact animals (initial d:r 3:1), in contrast to highly susceptible hamsters or deer mice, which transmit SARS-CoV-2 to each contact animal (d:r 1:1) within 5 days (4; B.D. Griffin et al., unpub. data, <https://doi.org/10.1101/2020.07.25.221291>). Our results suggest a tissue tropism for SARS-CoV-2 replication in bank voles to the upper respiratory tract, as seen for other species, such as ferrets, fruit bats, and raccoon dogs (3,7). The persistence of viral genome for at least 3 weeks in nasal tissue of directly inoculated animals was unexpected, especially because the last positive sample was retrieved 4 dpi from the respective bank voles (Table). This finding is most likely the result of the suspected clustering of SARS-CoV-2 infection foci in narrow

areas of the upper respiratory tract (L.M. Zaeck et al., unpub. data, <https://doi.org/10.1101/2020.10.17.339051>). Considering that virus isolation from these 21 dpi samples was not successful, the persistence of SARS-CoV-2 is unlikely to lead to the same shedding of infectious virus as it was shown previously for deer mice (A. Fagre et al., unpub. data, <https://doi.org/10.1101/2020.08.07.241810>; B.D. Griffin et al., unpub. data, <https://doi.org/10.1101/2020.07.25.221291>). Deer mice also seem to shed virus through the rectum. However, in bank voles, the SARS-CoV-2 genome could not be detected in the intestines. Although rectal swabs and fecal samples were qRT-PCR positive, the detected Cq values were high, indicating low viral RNA levels. Therefore, the detected viral RNA likely represents residues, which might have resulted from extensive grooming behavior and therefore do not correspond with actual virus shedding from the rectum or feces.

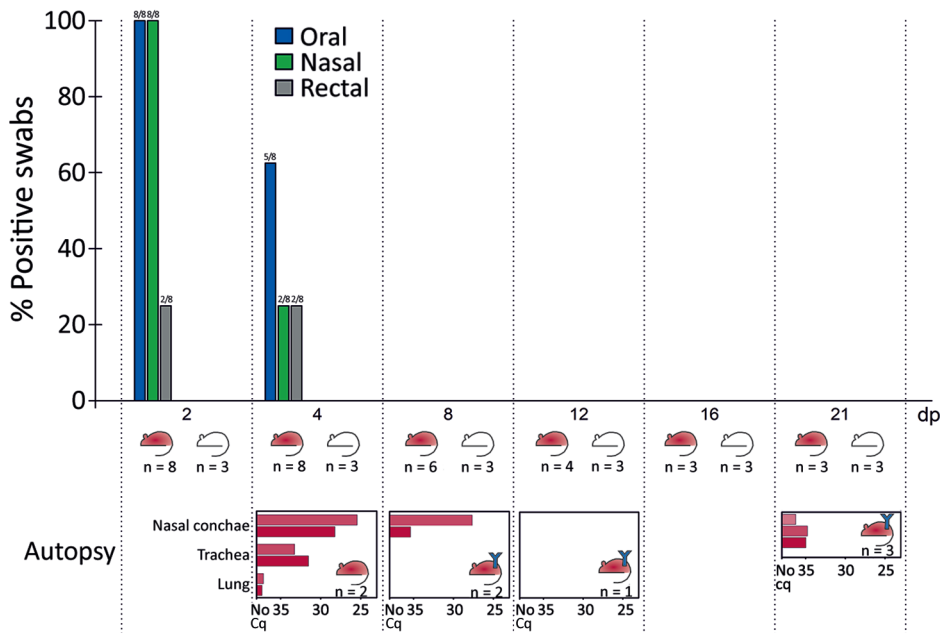


Figure. Percentage of swab specimens positive by quantitative reverse transcription PCR for SARS-CoV-2 on all sampling time points in study of experimental infection of bank voles. The red mouse symbols symbolize inoculated bank voles; the white mouse symbols represent co-housed contact bank voles. Blue Y symbols stand for detected antibodies against SARS-CoV-2 in the respective bank vole group. Quantitative reverse transcription PCR results for the sampled organs of the euthanized, inoculated bank voles are given below the main chart for each time point. Cq, quantification cycle; dpi, days postinoculation; n, number of bank voles; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

This study proves a general susceptibility of bank voles toward SARS-CoV-2 infection. However, bank voles did not transmit SARS-CoV-2 to contact animals, making them unlikely to maintain sustainable infection chains in nature. Therefore, the risk of bank voles becoming a reservoir for SARS-CoV-2 in nature (for example, after contact with infected cats) is low.

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The experimental protocol was assessed and approved by the ethics committee of the State Office of Agriculture, Food Safety, and Fisheries in Mecklenburg-Western Pomerania (permission no. MV/TSD/7221.3-2-010/18).

About the Author

Mr. Ulrich and Dr. Michelitsch are veterinarians and Mr. Halwe is a biologist at the Friedrich-Loeffler-Institut,

Greifswald-Insel Riems, Germany. Their research interests include pathogenesis and prevention of zoonotic viruses.

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Address for correspondence: Martin Beer, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany; email: martin.beer@fli.de

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Appendix

Animals and Housing Conditions

We obtained 8 female and 4 male bank voles, 7–9 weeks of age, from an in-house breeding colony at the Friedrich-Loeffler-Institut, Insel Riems, Germany. Prior to infection, we determined negative serologic status toward SARS-CoV-2 of the breeding colony by an indirect receptor-binding domain (RBD)-ELISA (*1*). All animals used in the trial tested RT-qPCR negative for SARS-CoV-2 on the day before infection by rhinarium, oral, and rectal swabs. For the duration of the study the animals were kept in individually ventilated cages (IVCs) with a light regime of 12 hours illumination and 12 hours darkness. Drinking water and a rodent diet were provided ad libitum. All handling procedures were performed under biosafety level 3 (BSL-3) conditions.

Study Design

We inoculated 9 bank voles with 1×10^5 tissue culture infection dose 50 (TCID₅₀) of the SARS-CoV-2 strain 2019_nCoV Muc-IMB-1 (GISAID ID_EPI_ISL_406862, designation hCoV-19/Germany/BavPat1/2020) by administering 70 μ L virus suspension to the nostrils and rhinarium. Inoculation took place under a short-term isoflurane-based inhalation anesthesia. Three inoculated bank voles were housed together in 1 IVC. Twenty-four hours after inoculation another 3 naïve in-contact bank voles, 1 per IVC, were co-housed with the directly inoculated animals. Physical examinations following a defined clinical score regarding general behavior, respiration, eyes, and neurologic symptoms were performed daily and bodyweight changes were monitored regularly (0, 2, 3, 4, 6, 7, 8, 9, 10, 12, 14, 16, and 21 days postinfection [dpi]). Oral, rhinarium, and cloacal swabs were taken from each animal at 2, 4, 8, 12, and 16 dpi. A fecal sample was taken from each IVC at these sampling points.

Two bank voles each were euthanized at 4 and 8 dpi and another at 12 dpi. At autopsy, a serum sample was collected and the nasal conchae, trachea, lung, heart, olfactory bulb, forebrain, cerebellum, liver, spleen, kidney, and small and large intestines were sampled. The remaining animals were euthanized at 21 dpi and serum samples were collected, as well as a sample of the nasal conchae.

Antibody Detection

Serum samples taken during euthanasia were tested by RBD-ELISA (1). Absorbance values >0.3 were considered antibody positive, those <0.2 antibody negative, and those in between as questionable (Appendix Table).

RNA Extraction and RT-qPCR

Before sampling, swabs (nerbe plus GmbH, <https://www.nerbe-plus.de>; Copan Italia S.p.A., <https://www.copangroup.com>) were dampened with Hank's 692 balanced salts (HBS) and Earle's balanced salts (EBS) in minimum essential medium (MEM). After sampling, the swabs were resuspended in 1 mL HBS and EBS MEM with the addition of penicillin and streptomycin. Fecal samples were directly collected in 1 mL of HBS and EBS MEM with the addition of penicillin and streptomycin. Organ samples were transferred in 1 mL of HBS and EBS MEM with an added steel bead and homogenized at 30,000 Hz for 2 minutes with the TissueLyserII (QIAGEN, <https://www.qiagen.com>). Nucleic acid was extracted from 100 μ L of the supernatant of all samples with the NucleoMag Vet kit (Macherey-Nagel, <https://www.mn-net.com>). Extracted viral RNA levels were determined by the already validated RT-qPCR nCoV_IP4, targeting the viral RNA-dependent RNA polymerase (2). We used a quantification cycle (Cq) value of 38 as a cutoff value (Appendix Table).

Virus Isolation

We attempted virus reisolation in cell culture on a Vero E6 cell line (L0929, collection of cell lines in veterinary medicine, Insel Riems, Germany) using HBS and EBS MEM with the addition of penicillin and streptomycin. Viral replication was determined by cytopathic effect within 72 hours after inoculation. Cultures with no visible cytopathic effect in the first passage were passaged once more.

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Appendix Table. RT-qPCR results from organ samples of all inoculated and contact bank voles as well as results from the indirect, multispecies ELISA*

dpi	Status	RBD ELISA	RT-qPCR quantification cycle (Cq)						
		absorbance/result	Nasal conchae	Trachea	Lung	Bulbus olfactorius	Cerebrum	Cerebellum	Spleen
4	Inoculated	0.01/negative	25.45	33.26	37.15	32.77	34.17	32.67	Neg
	Inoculated	0.01/negative	28.23	31.53	37.32	37.05	Neg	Neg	35.21
8	Inoculated	0.86/positive	27.66	Neg	Neg	Neg	Neg	Neg	Neg
	Inoculated	0.98/positive	35.38	Neg	Neg	Neg	Neg	Neg	Neg
12	Inoculated	1.02/positive	Neg	Neg	Neg	Neg	Neg	Neg	Neg
21	Inoculated	0.93/positive	36.25	ND	ND	ND	ND	ND	ND
	Inoculated	0.39/positive	34.78	ND	ND	ND	ND	ND	ND
	Inoculated	0.60/positive	34.97	ND	ND	ND	ND	ND	ND
	Contact	0.01/negative	Neg	ND	ND	ND	ND	ND	ND
	Contact	-0.00/negative	Neg	ND	ND	ND	ND	ND	ND
	Contact	-0.00/negative	Neg	ND	ND	ND	ND	ND	ND

*RT-qPCR results are given in quantification cycle values (Cq). dpi: days postinoculation; ND, not done; Neg, negative; RBD, receptor-binding domain.